Regulation of Cellular Proliferation and Intimal Formation following Balloon Injury in Atherosclerotic Rabbit Arteries

Robert D. Simari,* Hong San,* Mark Rekhter,‡ Takeshi Ohno,* David Gordon,‡ Gary J. Nabel,*‖** and Elizabeth G. Nabel*¶**
*Departments of *Internal Medicine, ‡Pathology, ‖Biological Chemistry, and ¶Physiology, and **Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan 48109*

Abstract

Injury to atherosclerotic arteries induces the expression of growth regulatory genes that stimulate cellular proliferation and intimal formation. Intimal expansion has been reduced in vivo in nonatherosclerotic balloon-injured arteries by transfer of genes that inhibit cell proliferation. It is not known, however, whether vascular cell proliferation can be inhibited after injury in more extensively diseased atherosclerotic arteries. Accordingly, the purpose of this study was to investigate whether expression of recombinant genes in atherosclerotic arteries after balloon injury could inhibit intimal cell proliferation. To test this hypothesis, we examined the response to balloon injury in atherosclerotic rabbit arteries after gene transfer of herpesvirus thymidine kinase gene (tk) and administration of ganciclovir. Smooth muscle cells from hyperlipidemic rabbit arteries infected with adenoviral vectors encoding tk were sensitive to ganciclovir, and bystander killing was observed in vitro. In atherosclerotic arteries, a human placental alkaline phosphatase reporter gene was expressed in intimal and medial smooth muscle cells and macrophages, identifying these cells as targets for gene transfer. Expression of tk in balloon-injured hyperlipidemic rabbit arteries followed by ganciclovir treatment resulted in a 64% reduction in intimal cell proliferation 7 d after gene transfer ($P = 0.004$), and a 35–49% reduction in intimal area 21 d after gene transfer, compared with five different control groups ($P < 0.05$). Replication of smooth muscle cells and macrophages was inhibited by tk expression and ganciclovir treatment. These findings indicate that transfer of a gene that inhibits cellular proliferation limits the intimal area in balloon-injured atherosclerotic arteries. Molecular approaches to the inhibition of cell proliferation in atherosclerotic arteries constitute a possible treatment for vascular proliferative diseases. (*J. Clin. Invest.* 1996. 98: 225–235.) Key words: gene transfer • thymidine kinase • adenoviral vectors • smooth muscle cells • macrophages

Introduction

Arterial injury induces the expression of growth factor and cytokine genes that stimulate vascular cell proliferation. Formation of a neointima is a regulated series of events, including an initial phase of smooth muscle cell replication, subsequent extracellular matrix synthesis, and followed by cell inhibition and connective tissue remodeling (reviewed in 1–3). Inhibition of smooth muscle cell replication by the local expression of recombinant genes can limit development of a neointima in balloon-injured arteries (4–8). One strategy to selectively eliminate dividing cells in injured arteries is to express a herpesvirus thymidine kinase (tk)[1] gene which phosphorylates the nucleoside analog, ganciclovir (GC), into a toxic form which leads to cell killing (9–12). Local delivery of recombinant adenoviral vectors encoding tk and exposure to ganciclovir has been shown to inhibit smooth muscle cell proliferation and decrease intimal formation in balloon-injured porcine and rat arteries (4–6). It is not known, however, whether these results can be achieved in extensively diseased atherosclerotic arteries. This issue has importance to clinical application of gene transfer for the treatment of vascular proliferative diseases, including atherosclerotic lesions. The potential of molecular genetic approaches to inhibit cell proliferation and limit intimal area in atherosclerotic arteries has not been explored. While previous studies have determined the feasibility of expressing reporter genes in hyperlipidemic rabbit and pig arteries (13, 14), the potential efficacy of therapeutic genes has not been reported. Accordingly, the purpose of this study was to investigate whether the proliferative response to arterial injury in atherosclerotic arteries can be inhibited by local expression of a tk gene followed by ganciclovir administration.

Methods

Recombinant adenoviral vectors. Three replication-deficient, recombinant adenoviral vectors were constructed, propagated, and purified as described (4, 15). These vectors were prepared from adenovirus-5 serotype and contain deletions in E1 and E3 regions, rendering them replication incompetent. The three adenoviral vectors (ADV) include a vector encoding herpesvirus thymidine kinase (ADV-tk), driven by a polyoma promoter and enhancer and containing an SV40 polyadenylation sequence. An adenoviral vector lacking a cDNA insert, ADV-ΔE1, was used for control experiments. A third adenoviral vector, ADV-hpAP, encodes for a human placental alkaline phosphatase (hpAP) reporter gene driven by a β-actin promoter and cytomegalovirus enhancer with an SV40 polyadenylation sequence. Viral stocks were sterilized with a 0.45-μm filter and evaluated for the presence of replication-competent virus by infection of 3T3 cells at an moi of 10. None of the stocks used in these experiments yielded replication-competent virus. Viral stocks were diluted to titers of 10^{10} plaque-forming units (pfu)/ml, stored at −20°C, and thawed on ice for 5 min before use.

In vitro testing of vectors in rabbit vascular smooth muscle cells. Primary vascular smooth muscle cells were obtained by explantation

Address correspondence to Elizabeth G. Nabel, 7301 MSRB III, Box 0644, University of Michigan, Ann Arbor, MI 48109-0644. Phone: 313-763-5103; FAX: 313-763-4851; E-mail: enabel@umich.edu

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1. *Abbreviations used in this paper:* ADV, adenoviral vectors; BrdC, 5-bromo-2′-deoxycytosine; GC, ganciclovir; hpAP, human placental alkaline phosphatase; tk, herpesvirus thymidine kinase.

from aortic segments of New Zealand White (NZW) (Hazleton Research Products, Denver, PA) rabbits fed a hyperlipidemic diet (0.5% cholesterol, 2.3% peanut oil) for 3 wk. At sacrifice, 3-cm abdominal aorta segments were immediately immersed in Medium 199 with 1% penicillin and streptomycin and 10% FCS. The lumenal surface of the aorta was scraped to remove endothelial cells, and the aortic segment was minced into fine pieces (< 1 mm) and placed in a 25-cm^2 flask coated with 1% gelatin and containing 2 ml media. The flask was tipped for 4 h so that the tissue would adhere to the gelatin. After 4 h, 10 ml media was added and the flask was kept flat in a 37°C incubator with 5% CO_2. Cells grew from the tissue within 7 d, and the aortic segments were removed. Smooth muscle cell identity was confirmed on second passage cells using a monoclonal antibody to smooth muscle α-actin (Sigma Chemical Co., St. Louis, MO) (16). To assess the sensitivity of ADV-tk–infected rabbit vascular muscle cells to GC, cells were grown to 30–40% confluence in Medium 199 with 10% FCS and infected with ADV-tk at an moi of 200 per cell. Previous estimates suggest that close to 100% of cells are transduced by these methods (15). 24 h later, the ADV-tk–infected cells were washed with media three times, harvested, mixed with noninfected cells in ratios from 0 to 100%, and plated at a density of 10,000 cells per well in a 96-well dish in eight replicates. The cells were incubated overnight at 37°C in a 5% CO_2 incubator to permit adherence to the plate, and the media was changed to either fresh media (−GC, four replicates) or media containing 5 μM ganciclovir (+GC, four replicates) (a generous gift from Syntex, Inc., Palo Alto, CA). Cell cultures were terminated at 6 d, and cell viability was determined using a colorimetric assay where cell viability is proportional to the absorbance at the test wavelength (570 nm) with subtraction of the reference wavelength (650 nm) (17).

Gene transfer in atherosclerotic arteries in vivo. NZW rabbits were sedated with ketamine (35 mg/kg i.m.) and xylazine (5 mg/kg i.m.) and intubated. Anesthesia was maintained with isoflurane. Before surgery, blood chemistries and serum cholesterol and triglyceride levels were measured (Roche Biomedical Laboratories, Nutley, NJ). Surgical exposure and arteriotomy of the right femoral artery was performed, and a 3–French Fogarty balloon catheter (Baxter Healthcare Corp., Mundelein, IL) was passed into the common iliac artery. The balloon was inflated in the right iliac artery and withdrawn three times. The right femoral artery was ligated distally, and the incision was closed. After surgery, the rabbits were fed a high fat diet consisting of 0.5% cholesterol and 2.3% peanut oil until they were killed. All animals received aspirin, 10 mg/kg, three times a week. Two rabbits were killed 3 wk after denuding injury and cholesterol feeding, and the iliac arteries were analyzed to determine the extent of atherosclerotic lesions.

3 wk after the first vascular injury, an angioplasty balloon injury was performed in the right iliac artery. Serum cholesterol and triglyceride levels were measured. A midline abdominal incision was made, and the distal aorta and iliac common arteries were isolated. Side branches in the iliac arteries were isolated and ligated. A 2–2.75-mm balloon angioplasty catheter (SciMed, BSC, Maple Grove, MN) was advanced via a distal aortotomy into the right iliac artery. The angioplasty balloon was inflated to six atmospheres of pressure for 1 min and deflated. Balloon inflation and deflation was repeated two times. Gene transfer was performed by withdrawing the balloon catheter to a position just proximal to the injury site. The arterial segment was isolated with temporary ligatures and rinsed with 5 ml of phosphate-buffered saline. Viral solution, 0.5 ml of 10^{10} pfu/ml (5×10^9 pfu total dose), was instilled within the injured segment at 150 mmHg pressure. After a 20-min incubation, the catheter was deflated, withdrawn, and the aortotomy site was repaired.

Gene transfer experiments were performed in 67 rabbits. Three additional rabbits underwent primary injury and lipid feeding, but were excluded due to arterial occlusion before secondary injury and infection. To determine which cells in the intima and media of atherosclerotic arteries express recombinant genes, seven rabbits were infected with ADV-hpAP vectors, and gene expression was analyzed 2 d later. To assess the therapeutic efficacy of a tk gene, ADV-tk or ADV-ΔE1 vectors or saline was instilled into injured atherosclerotic arteries in 60 rabbits (1 artery in each rabbit). Three animals died (one from each of the following groups: ADV-ΔE1/+GC, ADV-tk/−GC, and noninfected/+GC) within 3 d perioperatively from distal aortic occlusion, a complication of surgical repair, and were excluded from further analysis. 36 h after balloon injury and adenoviral infection or saline infusion, animals were randomized to treatment with GC, 50 mg/kg per d, or a weight-adjusted equivalent volume of saline was administered intravenously for 6 d. The effect of tk expression and GC treatment on cell proliferation was evaluated 7 d after angioplasty and ADV-tk infection in eight rabbits. These rabbits received i.v. infusions of 5-bromo-2′-deoxycytosine (BrdC) 1, 18, and 24 h before death. Immunohistochemistry with a monoclonal antibody to BrdC was performed to label nuclei in proliferating cells. Intimal and medial areas were measured 21 d after gene transfer in 49 rabbits, divided into six treatment groups: noninfected/-GC (n = 5), noninfected/+GC (n = 5), ADV-ΔE1/-GC (n = 10), ADV-ΔE1/+GC (n = 9), ADV-tk/−GC (n = 9), and ADV-tk/+GC (n = 11).

At death, tissue samples were obtained from the treated right iliac artery, brain, carotid artery, heart, lung, liver, spleen, kidney, lower limb skeletal muscle, and ovary. Each adenovirus-infected artery was processed in an identical manner by division into four cross sections. Sections 1 and 3 were fixed in methyl Carnoy's solution and sections 2 and 4 were fixed in 10% buffered formalin. Organ specimens were fixed in formalin. All specimens were paraffin embedded and sections were stained with hematoxylin and eosin. In addition, samples of blood were obtained before gene transfer and at death for biochemical analysis. All animal experiments were performed in accordance with National Institutes of Health guidelines and with the approval of the University of Michigan Committee in the Use and Care of Animals.

Histochemistry. Expression of recombinant hpAP protein was detected by histochemical analysis of infected balloon-injured atherosclerotic arteries. 2 d after adenoviral infection, artery specimens were fixed in 10% buffered formalin for 16 h, placed in 70% ethyl alcohol, and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 6 μm onto poly-L-lysine slides. The slides were deparaffinized in three changes of xylene, rehydrated in 100%, 95%, and 75% ethyl alcohol, and incubated in PBS at 65°C for 60 min to inactivate endogenous alkaline phosphatase. The sections were incubated in PBS containing a chromogenic substrate of 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine (1 mg/ml, GIBCO BRL, Gaithersburg, MD) and nitroblue tetrazolium chloride (1 mg/ml, GIBCO BRL) for 19 h. This substrate yields a dark blue purple stain in the presence of alkaline phosphatase. Sections were rinsed with PBS and counterstained with methyl green.

Immunohistochemistry. Immunohistochemical studies were performed to identify smooth muscle cells, macrophages, and proliferating cells with smooth muscle α-actin, RAM-11, and BrdC antibodies, respectively, using methods as previously described (4, 16, 18, 19). Briefly, serial sections of methyl Carnoy or formalin-fixed, paraffin-embedded sections (6 μm) were placed in poly-L-lysine–coated slides, deparaffinized in three changes of xylene, and rehydrated in 100%, 95%, and 75% ethyl alcohol. Incubation in 0.3% hydrogen peroxide for 30 min exhausted endogenous peroxidase activity. For each antibody, the dilution that yielded optimal specific staining was determined in pilot experiments. The following primary antibodies were used: a monoclonal mouse antismooth muscle α-actin antibody, 1:500 dilution (Boehringer Mannheim Biochemical Div., Indianapolis, IN); a rabbit macrophage-specific antibody RAM-11, 1:100 dilution (Dako Corp., Santa Barbara, CA); and a monoclonal mouse anti–BrdC antibody, 1:1,000 dilution (Amersham Corp., Arlington Heights, IL). Control experiments were performed using purified mouse IgG_{2b} antibody, 1:100 dilution (Promega Corp., Madison, WI), which did not stain the arterial specimens. Slides were developed with either a streptavidin-horseradish peroxidase complex (Vector Laboratories Inc., Burlingame, CA), or a Vectastain ABC-alkaline phosphatase reagent (Vector Laboratories Inc.), and counterstained in methyl green. The streptavidin-horseradish peroxidase complex, 1:5,000 dilution, was applied for 30 min at room temperature followed by a diaminobenzidine substrate (Sigma Chemical Co.) in 0.045% nickel chloride (room temperature,

10 min) to produce a gray-black reaction and by counterstain with methyl green. Biotinylated anti-mouse immunoglobulin, 1:200 dilution (Vector Laboratories Inc.), was applied for 30 min, followed by another 30-min incubation in a Vectastain ABC-alkaline phosphatase reagent. The substrate 3-amino-9-ethyl-carbazole yielded a red reaction product and sections were counterstained in methyl green.

To identify the cell types expressing the transgene hpAP, combined alkaline phosphatase histochemical staining and immunocytochemistry was performed. After alkaline phosphatase staining, slides were washed in PBS and incubated with the primary antibody: a monoclonal mouse antismooth muscle α-actin antibody, 1:500 dilution; or a rabbit macrophage-specific antibody RAM-11, 1:250 dilution, using 2% normal horse serum as diluent for 1 h at room temperature. The secondary antibody (biotinylated horse anti–mouse antibody, 1:200 dilution, Vector Laboratories Inc.) was incubated for 30 min at room temperature, followed by streptavidin-colloidal gold (1:1,000 dilution, Goldmark Biologicals, Phillipsburg, NJ) for 30 min. Slides were then copiously washed in distilled water and incubated with the silver enhancement kit (Goldmark Biologicals) for 10 to 15 min under microscopic control. This step produced black-silver grains over the cytoplasm. Methyl green counter stain was used to visualize nuclei.

Measurement of efficacy of gene transfer and cell proliferation and morphometry. To investigate the efficiency of adenoviral infection in balloon-injured, atherosclerotic rabbit arteries, arterial specimens were subjected to histochemical staining for alkaline phosphatase. A microscope-based video image analysis system (Image One System; Universal Imaging Corp., West Chester, PA) was used to count the total number of cells and the total number of alkaline phosphatase-positive cells in the intima and media of four cross sections in each artery that spanned the 1.5-cm region of arterial injury and adenoviral infection. Measurements were made in a blinded manner by two independent observers. Transfection efficiency was calculated as the ratio of alkaline phosphatase-positive cells per total number of cells in the intima and media of each artery cross section.

To measure the number of proliferating cells, arteries were labeled with BrdC and immunohistochemistry was performed. The total number of nuclei and BrdC-positive nuclei in the intima and media of experimental and control arteries was counted in a blinded fashion by quantitative morphometry (Image One Systems; Universal Imaging Corp.). A proliferation index was calculated as the ratio of BrdC-positive cells to total number of cells. To identify the cell types incorporating BrdC, double immunohistochemical staining was performed using antibodies to BrdC and α-actin and to BrdC and RAM-11. The number of cells double labeled for BrdC and smooth muscle α-actin and RAM-11 were measured by the criteria that the borders of BrdC-positive nuclei were greater than 50% contiguous with α-actin and RAM-11 cytoplasmic staining. In each artery cross section, eight random high power fields were measured. The eight high power fields from each artery were averaged to obtain a measure for each artery.

Measurements of intimal and medial cross-sectional area were determined by two independent reviewers in a blinded fashion on four sections from each artery that spanned the 1.5-cm region of arterial injury and adenoviral infection using the image analysis system. Intimal and medial boundaries were determined by digital planimetry. The four measurements were averaged for each artery. Interobserver variability was ~2%.

Analysis of organ toxicity. Organ specimens from noninfected tissues, including brain, carotid artery, heart, lung, liver, spleen, kidney, ovary, and skeletal muscle, were obtained at death, formalin-fixed and paraffin embedded, and sections were stained with hematoxylin and eosin. Arterial segments from adenovirus-infected arteries were compared to noninfected arteries 3 wk after gene transfer to assess possible inflammation and other pathology. Infected arterial specimens from each of the treatment groups were also compared. Each artery and organ specimen was examined by an experienced pathologist (D. Gordon) in a blinded fashion.

Statistical analysis. All values are expressed as mean±SEM. Comparisons of intimal and medial BrdC labeling index were made by two

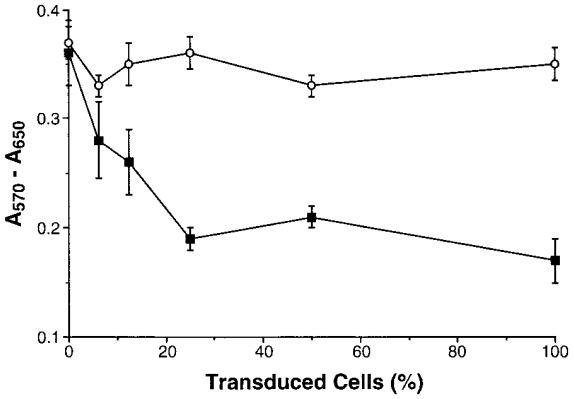

Figure 1. In vitro sensitivity of rabbit vascular smooth muscle cells to GC after infection with ADV-tk. Cells were grown to 30–40% confluence and infected with ADV-tk at an moi of 200 per cell. Infected cells were mixed with noninfected cells in the ratio of transfected to untransfected cells as indicated on the x-axis, and treated with fresh media (−GC) or media containing 5 μM ganciclovir (+GC). Cell proliferation was determined using a colorimetric assay. Data are expressed as mean±SEM. Four replicate experiments were performed. Circles = −GC; squares = +GC.

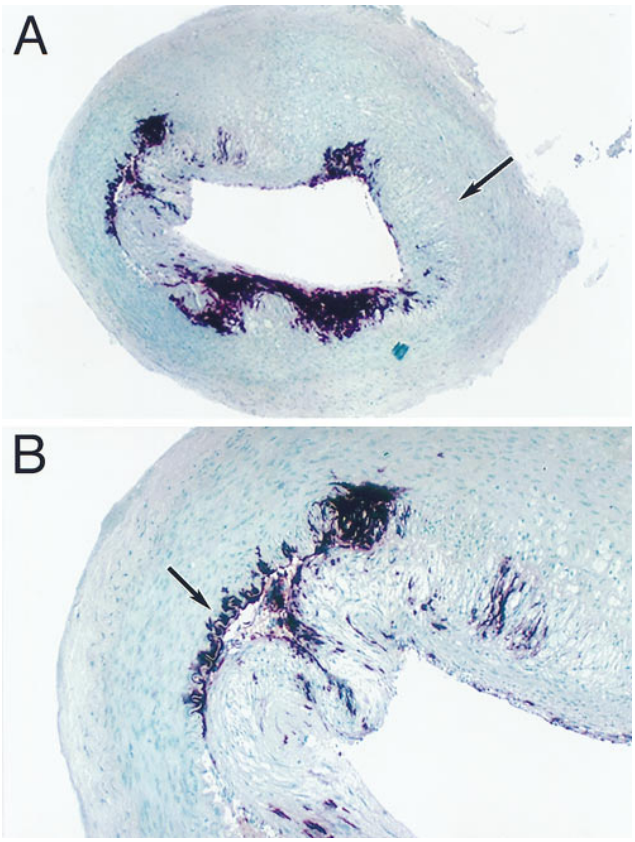

Figure 2. Gene expression in atherosclerotic arteries 2 d after balloon injury and ADV-hpAP infection. (*A*) Alkaline-phosphatase expression was observed in the intima and media of balloon-injured atherosclerotic arteries. Arrow denotes internal elastic lamina. (*B*) Gene expression in the media was prominent along a dissection plane where the internal elastic lamina was fractured, as designated by the arrow. Magnification: *A* ×44 and *B* ×97.

tailed, unpaired *t* test. Comparisons of intimal and medial areas were made by ANOVA with Dunnett *t* test. Statistical significance was assumed if a null hypothesis could be rejected at the 0.05 level.

Results

In vitro sensitivity of rabbit vascular smooth muscle cells to ganciclovir after infection with ADV-tk. The efficacy of tk expression in limiting rabbit smooth muscle cell proliferation was first assessed in vitro. Primary rabbit smooth muscle cells from atherosclerotic arteries were infected with ADV-tk vectors (moi of 200/cell) and exposed to GC or control media. Cells infected with ADV-tk and not treated with GC remained viable (Fig. 1). In contrast, cells infected with ADV-tk and treated with GC were nonviable 6 d later. Mixtures of ADV-tk–infected and –noninfected cells demonstrated that as few as 10% of in-

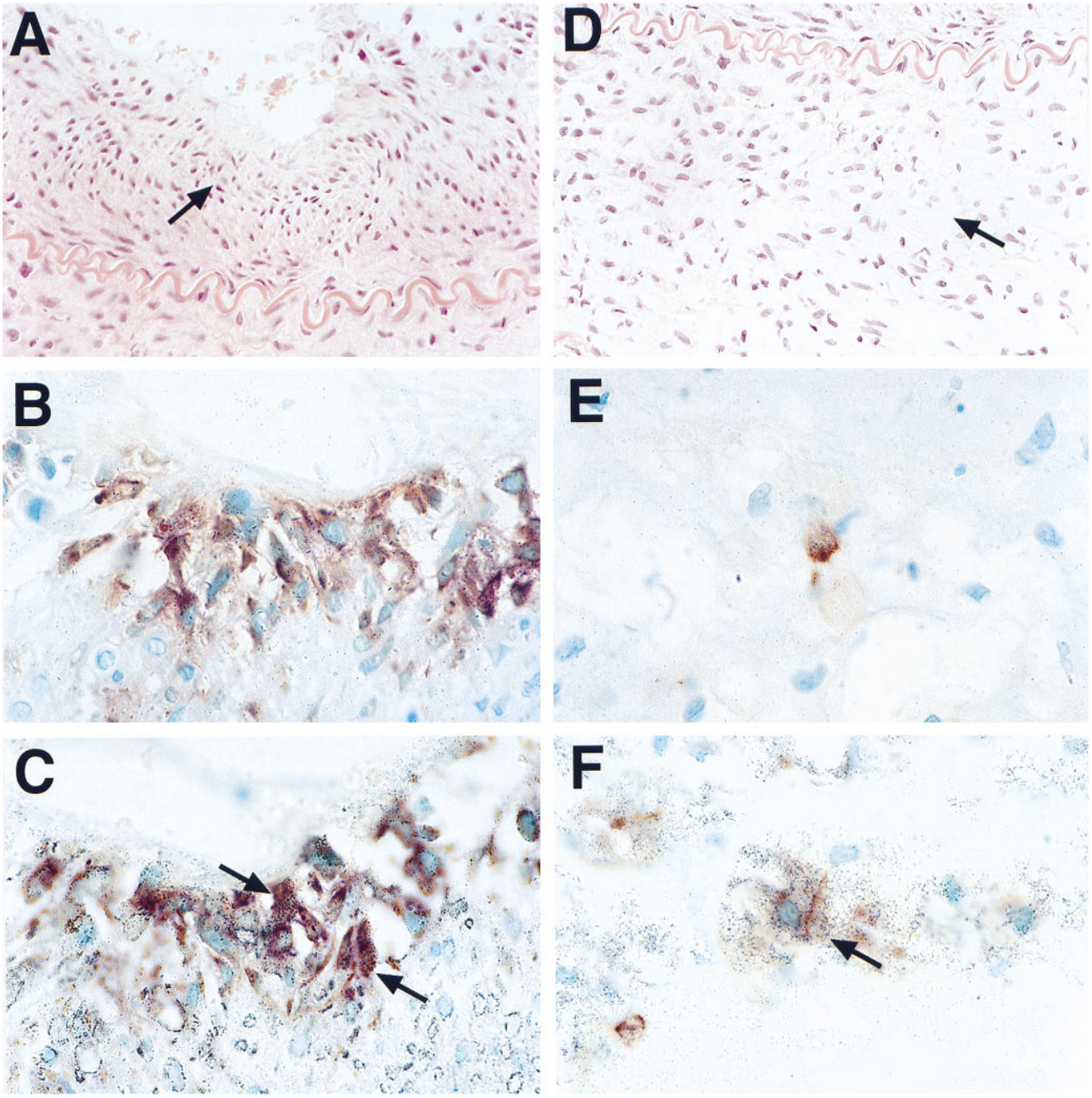

Figure 3. Alkaline-phosphatase expression in smooth muscle cells and macrophages in atherosclerotic arteries. (*A*) Hematoxylin and eosin staining of intimal lesion. The arrow denotes area shown in *B* and *C*. (*B*) Alkaline-phosphatase expression in intimal cells with horse serum control for immunostaining. (*C*) Intimal cells double labeled with alkaline-phosphatase staining and smooth muscle cell α-actin immunostaining. Arrows denote double-labeled positive cells. (*D*) Hematoxylin and eosin staining of media adjacent to the intima shown in *A*. The arrow denotes area shown in *E* and *F*. (*E*) Alkaline-phosphatase expression in medial cells with horse serum control for immunostaining. (*F*) Medial cells which double label with alkaline-phosphatase staining and RAM 11 immunostaining. Arrows denote double-labeled positive cells. Magnification: *A* and *D* ×200 and *B*, *C*, *E*, and *F* ×725.

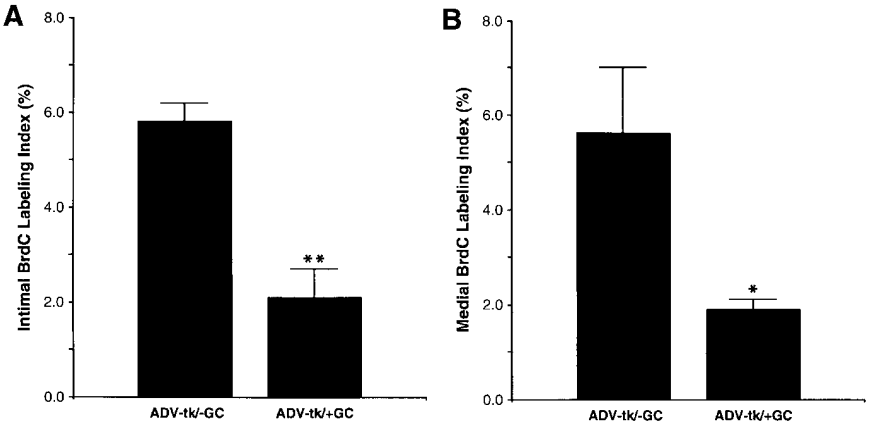

Figure 4. Inhibition of intimal and medial cell DNA synthesis by ADV-tk/+GC after arterial injury. Hyperlipidemic rabbit arteries were injured by balloon angioplasty, infected with ADV-tk (total dose 5×10^9 pfu), and treated with saline (−GC, $n = 4$ arteries) or GC (+GC, $n = 4$ arteries). 7 d later, animals were infused with BrdC and arteries were immunostained with a monoclonal antibody specific for BrdC. Total nuclei and BrdC-positive nuclei were quantitated. (*A*) Intimal cell BrdC incorporation index. (*B*) Medial cell BrdC incorporation index. Data are expressed as mean±SEM. *$P = 0.04$, **$P = 0.004$, unpaired, two-tailed *t* test.

fected cells conferred susceptibility to GC to the entire population of cells, thus demonstrating a bystander effect in killing adjacent, noninfected cells.

Recombinant gene expression in atherosclerotic arteries after adenoviral gene transfer. Atherosclerotic lesions were produced in rabbit iliac arteries by subjecting the arteries to a primary denuding injury with a 3 French Fogarty catheter, followed by 3 wk of lipid feeding. This diet produced an increase in serum cholesterol levels from 54±4 mg/dl to 1,107±44 mg/dl and in triglyceride levels from 36±1 mg/dl to 218±15 mg/dl ($n = 67$). Examination of injured, noninfected iliac arteries (3 wk after lipid feeding) by light microscopy in two rabbits revealed a thickened intima with foam cells and smooth muscle cells, consistent with atherosclerotic lesions (20, 21).

To determine whether expression of recombinant genes could be achieved in these atherosclerotic arteries in vivo, the hyperlipidemic arteries were then reinjured at the previous site of denudation with a balloon angioplasty catheter and infected with adenoviral vectors encoding a reporter gene, hpAP (5×10^9 pfu, total dose). Infection of these arteries with ADV-hpAP resulted in gene expression in the intima and the media (Fig. 2 *A*). Gene expression was also found in medial regions when the internal elastic lamina was dissected or ruptured (Fig. 2 *B*), suggesting that adenoviral vectors may track along dissection planes and infect cells in deeper regions of the arterial wall. The ability to infect these layers may be of importance since cells in these regions of the media may contribute to cellular proliferation and intimal hyperplasia (22, 23).

The efficiency of gene transfer in these atherosclerotic arteries was then quantified. The ratio of alkaline phosphatase–positive cells to the total number of cells was calculated for each arterial cross section, and the ratios were averaged to determine expression in the intima and the media. Expression of alkaline phosphatase in the intima and media was variable, ranging from 4.2% to 69.1% (32.0±9.4%, mean±SEM) of cells in the intima and 0% to 45.9% (mean 19.6±6.3%, mean±SEM) of cells in the media. When the number of alkaline phosphatase positive cells and the total number of cells in the intimal and media were combined, then expression for the arterial cross section (excluding adventitia) ranged from 2.7% to 52.7% (23.5±7.0%, mean±SEM).

Double-label staining using histochemical staining for alkaline phosphatase and immunohistochemical staining for specific cell types was performed to determine the phenotype of genetically modified cells. As noted previously in balloon-injured normal arteries (4), alkaline phosphatase staining was observed in smooth muscle cells as determined by reactivity with antismooth muscle α-actin antibody (Fig. 3, *A–C*). In contrast to the pattern of expression observed in normal arteries, recombinant gene expression was also seen in macrophages, as defined by reactivity with an antibody specific for rabbit macrophages, RAM-11 (Fig. 3, *D–F*). The majority of hpAP-positive cells, however, stained for smooth muscle α-actin. These findings suggest that predominantly smooth muscle cells, but also macrophages, express recombinant genes in atherosclerotic lesions, and that these vascular cells could be used to regulate the response to injury in vivo.

Expression of tk and ganciclovir administration inhibits cell proliferation in atherosclerotic arteries in vivo. To investigate the effects of tk/GC on cell proliferation in atherosclerotic arteries after balloon injury, right iliac arteries underwent primary denudation followed by 3 wk of lipid feeding. These hyperlipidemic arteries were reinjured at the denudation site with a balloon angioplasty catheter and infected with 5×10^9 pfu (total dose) ADV-tk. 36 h after injury and infection, the rabbits were randomized to treatment with either GC or saline for 6 d. Cell proliferation was assessed 7 d after ADV-tk infection by quantitating incorporation of BrdC into intimal and medial cells. ADV-tk infection plus GC treatment significantly inhibited intimal and medial cell DNA synthesis (Fig. 4). A 64% reduction in intimal BrdC incorporation was observed in ADV-tk/+GC arteries compared with ADV-tk/−GC arteries (2.1±0.6% vs 5.8±0.4%, $P = 0.004$). A similar reduction in medial BrdC incorporation (66%) was observed in ADV-tk/+GC arteries compared with ADV-tk/−GC arteries (1.9±0.2% vs 5.6± 1.4%, $P = 0.04$).

We next examined whether tk expression and GC treatment preferentially eliminated certain proliferating cell types. Quantitative morphometry was used to measure the cell types which stained BrdC positive. In ADV-tk/−GC rabbits, 51.1± 1.2% of intimal BrdC-positive cells were smooth muscle α-actin positive and 18.2±3.9% of intimal BrdC-positive cells were RAM-11 positive. In ADV-tk/+GC rabbits, 60.2±3.5% of BrdC-positive cells in the intima were smooth muscle α-actin positive and 16.9±5.8% of intimal BrdC-positive cells were RAM-11 positive. The 30.7% (ADV-tk/−GC) and 22.9% (ADV-tk/+GC) of labeled cells that were not accounted for as clearly being smooth muscle cells or macrophages may have been lymphocytes, endothelial cells, α-actin–negative smooth muscle cells, or RAM-11–negative macrophages, as our meth-

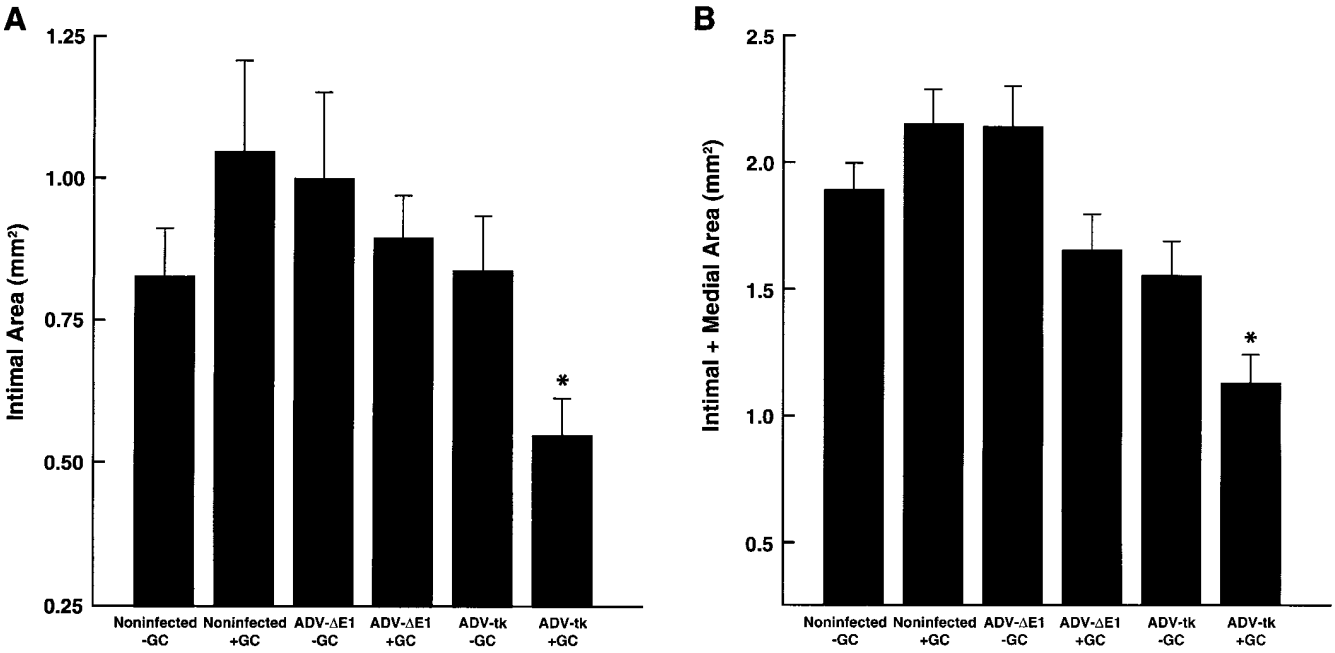

Figure 5. Effects of ADV-tk/+GC on intimal and medial areas after arterial injury. Rabbits underwent a denudation injury, lipid feeding, and balloon-angioplasty injury (6 atm, 3 min), followed by adenoviral gene transfer with ADV-tk or ADV-ΔE1 or infusion with saline (noninfected). Animals were randomized to receive systemic saline or GC therapy IV for 6 d. Artery segments were excised 21 d after gene transfer, and intimal and medial cross-sectional areas were measured. Intimal areas from the six groups are shown in *A*, and intimal and medial areas are shown in *B*. Data are expressed as mean±SEM. $*P < 0.05$, ADV-tk/+GC vs five control groups, ANOVA with Dunnett *t* test.

ods may have underestimated α-actin– and RAM-11–positive cells. The similar cellular distribution in the two groups, in spite of decreased total numbers of proliferating cells, suggests that both smooth muscle cells and macrophages proliferate in atherosclerotic lesions in these rabbits, and both cell types are susceptible to killing in a parallel manner by expression of a tk gene and ganciclovir.

Expression of tk and ganciclovir administration reduces intimal hyperplasia in atherosclerotic arteries in vivo. The size of intimal and medial lesions in atherosclerotic arteries of hyperlipidemic rabbits was measured after second balloon injury and treatment with saline, or infection with ADV-ΔE1 or ADV-tk vectors (5×10^9 pfu, total dose). Six groups were studied: noninfected/-GC ($n = 5$ arteries), noninfected/+GC ($n = 5$ arteries), ADV-ΔE1/-GC ($n = 10$ arteries), ADV-ΔE1/+GC ($n = 9$ arteries), ADV-tk/−GC ($n = 9$ arteries), and ADV-tk/+GC ($n = 11$ arteries). 21 d later, intimal and medial areas were measured by digital planimetry. A significant reduction in intimal area was observed in ADV-tk arteries treated with GC (ADV-tk/+GC, 0.54±0.06 mm^2) compared with ADV-tk arteries treated with saline (ADV-tk/−GC, 0.83±0.09 mm^2), noninfected arteries, (noninfected/-GC, 0.82±0.08 mm^2; noninfected/+GC, 1.04±0.16 mm^2), and ADV-ΔE1 arteries treated with GC or saline (ADV-ΔE1/-GC 1.00±0.15 mm^2; ADV-ΔE1/+GC, 0.90±0.07 mm^2, $P < 0.05$; ADV-tk/+GC vs five control groups, ANOVA with Dunnett *t* test) (Figs. 5 and 6). There was no independent effect of adenoviral infection or ganciclovir treatment on intimal area. There was a decrease in medial area in the ADV-tk/+GC arteries, but this difference was not statistically different compared with the five other groups. Medial areas were: ADV-tk/−GC, 0.71±0.06 mm^2; ADV-tk/+GC, 0.58±0.04 mm^2; ADV-ΔE1/-GC, 0.83± 0.08 mm^2; and ADV-ΔE1/+GC, 0.75±0.07 mm^2; and noninfected/-GC 0.87±0.09 mm^2; noninfected/+GC 0.83±0.09 mm^2 (P = NS). This decrease in medial area was not unexpected given a decrease in medial cell proliferation. When the intimal and medial areas were combined, there was a significant decrease in intimal and medial wall thickness compared with the five other groups ($P < 0.05$, ANOVA with Dunnett *t* test) (Fig. 5 *B*). Thus, localized infection of atherosclerotic arteries with an adenovirus encoding tk after arterial injury and systemic GC treatment resulted in a significant reduction in intimal lesion size.

Effects of adenoviral infection on rabbit arteries and other tissues. In this study, adenoviral vectors are delivered by a catheter to a localized region of the artery. To determine whether release of residual virus into the arterial circulation could cause toxicity in tissues in vivo, we examined noninfected and infected arteries and other major organs by histopathology. Analysis of arteries revealed no major cytopathology in response to ADV-tk, ADV-ΔE1 or ADV-hpAP vectors, or saline. Occasional mononuclear inflammatory cells were present in injured, noninfected, and infected atherosclerotic rabbit arteries, but there were no differences in the number of inflammatory cells in arteries receiving adenoviral vectors compared with atherosclerotic arteries not treated with adenoviral vectors. Likewise, there was no evidence of necrosis or increased inflammation in ADV-tk/+GC arteries compared with ADV-tk/−GC, ADV-ΔE1/+GC, ADV-ΔE1/-GC, or noninfected arteries. Analysis of organs, including brain, carotid artery, heart, lung, liver, spleen, kidney, skeletal muscle, and gonadal tissue showed minor findings such as hemosiderin in the spleen and megakaryocytic nuclei in the kidney. There were no differences in the incidence of these incidental find-

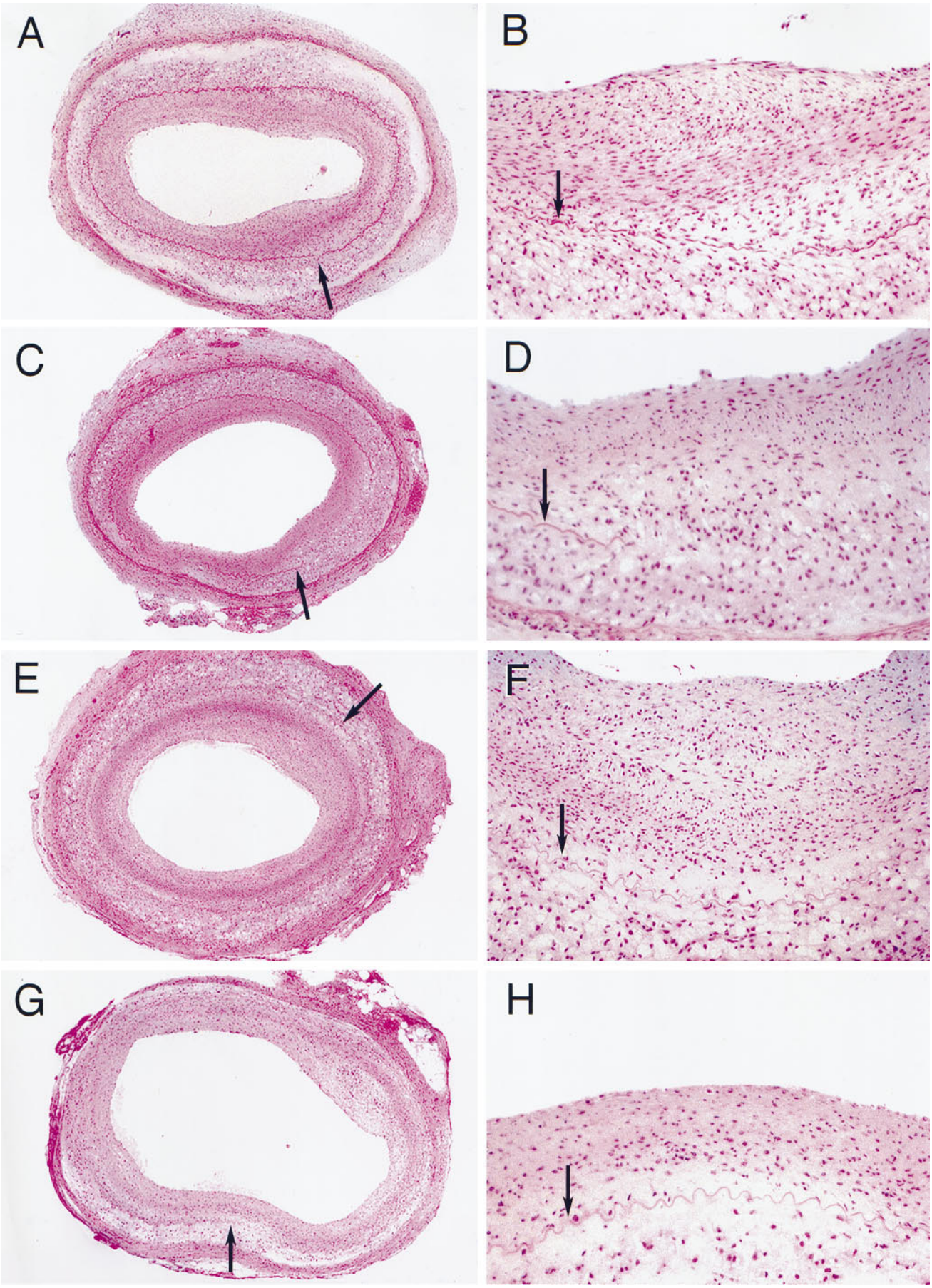
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Table I. Organ Pathology After Local Adenoviral Infection of Iliac Arteries

Organ	Findings	Frequency			
		ADV-ΔE1		ADV-tk	
		−GC	+GC	−GC	+GC
Brain	abnormalities not observed	—	—	—	—
Carotid artery	abnormalities not observed	—	—	—	—
Heart	abnormalities not observed	—	—	—	—
Lung	(a) occasional eosinophils	2/5	0/5	3/9	4/11
	(b) chronic interstitial infiltrates	0/5	0/5	0/9	2/11
Liver	(a) rare mononuclear inflammatory cells	1/5	1/5	3/9	1/11
	(b) eosinophils present	0/5	0/5	1/9	0/11
	(c) fatty changes	0/5	0/5	0/9	2/11
Spleen	hemosiderin deposits	5/5	4/5	9/9	10/11
Kidney	occasional multinucleate cells	5/5	2/5	5/9	4/11
Ovary	focal calcifications	1/5	0/5	1/9	3/11
Skeletal muscle	abnormalities not observed	—	—	—	—

ings among the virally infected groups (Table I), and these findings have been previously observed in NZW rabbits exposed to nonviral vectors (24). Serum biochemical parameters were within normal limits, aside from the elevations in serum cholesterol and triglycerides. Thus, adenoviral vectors administered intraarterially via a catheter to atherosclerotic rabbit arteries were not associated with significant systemic toxicities.

Discussion

Arterial injury stimulates a series of proliferative, inflammatory, and thrombotic responses. Under normal circumstances, arterial endothelial and smooth muscle cells respond to pathological stimuli with a reparative process. In atherosclerotic arteries, this protective response consists of the formation of fibrous and fatty lesions accompanied by inflammation. However, arterial injuries can lead to excessive fibroproliferative and inflammatory lesions that produce vessel occlusion, leading to myocardial, cerebral, and peripheral ischemia. Vascular cell replication plays a central role in the pathogenesis of atherosclerosis and intimal expansion after arterial injury. Our ability to target dividing smooth muscle and other vascular cells provides the opportunity to investigate the pathogenesis of lesion formation and to develop therapeutic agents to limit lesion size.

Smooth muscle cell replication can be inhibited by elimination of dividing cells and/or by targeting specific gene products that regulate cell division, such as cell cycle proteins. Recently, a number of studies have investigated molecular genetic approaches to inhibit vascular cell proliferation in nonatherosclerotic arteries in vivo (4–8, 25–31). Despite considerable success in these models, it remained unclear whether vascular cell proliferation could be inhibited in extensively diseased atherosclerotic arteries. Furthermore, although previous studies have demonstrated the feasibility of gene transfer to atherosclerotic rabbit arteries (13, 14), a recent study suggested that atherosclerosis might reduce the transfection efficiency achieved with adenoviral vectors compared to nonatherosclerotic arteries, and thus might potentially limit arterial gene therapy (13).

In this study, the effects of tk gene expression and systemic ganciclovir treatment on intimal lesion formation in injured atherosclerotic arteries has been analyzed. We observed expression of a reporter gene, alkaline phosphatase, in intimal and medial smooth muscle cells and macrophages 2 d after balloon injury and adenoviral infection, suggesting that the appropriate cell types could be transduced in vivo. Interestingly, gene expression was observed in the media along dissection planes resulting from fracture of the internal elastic lamina. Rabbit smooth muscle cells derived from hyperlipidemic aorta were infectable by ADV-tk vectors and conferred susceptibility to ganciclovir to noninfected smooth muscle cells in culture, demonstrating a bystander effect. Thymidine kinase gene transfer followed by ganciclovir treatment in atherosclerotic arteries reduced intimal expansion in vivo after arterial injury. No independent effects of adenoviral infection or ganciclovir treatment were observed on intimal area. These findings are not surprising given the lack of effect of adenoviral infection or GC treatment on injured arteries in previous studies (4–6).

These findings have a number of basic and clinical implications. The effects on intimal cell proliferation are likely due to inhibition of DNA replication after phosphorylation of ganciclovir by the enzyme thymidine kinase. The lethal effects of phosphorylated ganciclovir are not likely limited to transduced cells; tk-negative smooth muscle cells were killed in vitro when the population of cultured cells contained only 10% tk-positive cells. These results suggest that not all cells need to be trans-

Figure 6. Representative photomicrographs demonstrating inhibition of intimal area by ADV-tk/+GC after arterial injury. Balloon-injured, hyperlipidemic rabbit arteries were infected with ADV-tk or ADV-ΔE1 and randomized to treatment with saline (−GC) or ganciclovir (+GC). Sections were obtained for analysis 21 d after arterial injury and gene transfer. Sections from ADV-ΔE1/-GC (*A* and *B*), ADV-ΔE1/+GC (*C* and *D*), ADV-tk/−GC (*E* and *F*), and ADV-tk/+GC (*G* and *H*) are shown. Black arrows in *A*, *C*, *E*, and *G* delineate the magnified region shown in *B*, *D*, *F*, and *H*, respectively. Black arrows in *B*, *D*, *F*, and *H* indicate the internal elastic lamina. Magnification: *A*, *C*, *E*, *G* ×44; *B*, *D*, *F*, *H* ×156.

duced in vivo to inhibit cell replication but rather, inhibition of cell proliferation can be achieved by a paracrine mechanism. It has been postulated that a metabolite of phosphorylated ganciclovir is transferable to adjacent, nontransduced dividing cells, where incorporation into DNA induces chain termination and cell death in these nontransduced cells (10). The exact mechanism by which this transfer occurs in vascular cells is unknown. Previous studies in tumor cells transduced with retroviral vectors expressing tk have suggested that the bystander effect appears to be related to apoptotic cell death and/or metabolic cooperation between cells (32, 33). Flow cytometry and electron microscopy studies suggest that apoptotic vesicles produced from tk-positive tumor cells can be phagocytized by adjacent, unmodified tumor cells. In addition, other studies have demonstrated cell contact and transfer of labeled ganciclovir between tk-positive and tk-negative cells in culture, suggesting that a ganciclovir metabolite product can mediate cytotoxicity as a result of direct contact, perhaps via gap junctions. It is unclear whether the bystander effect is functional between dissimilar cell types, as in an atherosclerotic artery. Ongoing studies are examining the mechanism of this bystander effect in vascular cells.

This prodrug approach appears well-suited to address the problem of vascular cell proliferation in injured arteries. Adenoviral infection of arteries produces sustained gene expression for $\sim$ 2 wk (13–15, 34–37), and conversion of a prodrug to a toxic form during the peak of cell proliferation after balloon injury appears to provide local concentrations sufficient to reduce cell proliferation within the first week after arterial injury and limit intimal size at later time points (4–6). It is known that gene transfer is feasible in atherosclerotic arteries (13, 14); however, it was unknown whether this approach would be therapeutic. Previous studies using percutaneous delivery of adenoviral vectors encoding a *lacZ* reporter gene in atherosclerotic rabbit arteries with a channel balloon and a 90–150-s infusion demonstrated gene expression in $\sim$ 0.2% of intimal and medial cells. This result was approximately tenfold lower than observed in balloon-injured normal arteries ($\sim$ 2.1% of intimal and medial cells) (13). In addition, β-galactosidase activity was observed only in smooth muscle cells and not macrophages determined by cell specific immunostaining. In other studies, no differences in luciferase activity were observed between atherosclerotic balloon-injured or normal porcine coronary arteries after percutaneous delivery of adenoviral vectors encoding a luciferase gene with a porous balloon catheter, and transfection efficiencies in hyperlipidemic porcine arteries were not determined (14). In this study, we observed alkaline phosphatase expression in 4.2% to 69.1% of intimal cells and 0% to 45.9% of medial cells in these balloon-injured atherosclerotic rabbit iliac arteries. In addition, we observed alkaline phosphatase staining in smooth muscle cells and macrophages determined by cell type–specific immunostaining of double-labeled sections. The range in transfection efficiency and cell types expressing recombinant genes in atherosclerotic rabbit arteries observed between studies may be attributable to technical issues, including choice of reporter gene, sensitivity of assay to detect gene expression, catheter design and delivery features, possible run-off of vector by side branches, duration of instillation, and a percutaneous or surgical approach. We instilled viral solutions into contained iliac segments for 20 min after ligature of perforating branches to optimize delivery of vectors and gene expression. While this approach may not mimic percutaneous procedures in their present form, such technical modifications can be adapted to facilitate clinical applications. The findings in this study suggest that atherosclerosis, per se, does not preclude effective inhibition of cell proliferation and reduction of intimal lesion size after adenovirus-mediated gene transfer.

Immunostaining with BrdC antibodies and cell type–specific monoclonal antibodies revealed that $\sim$ 50–60% of labeled cells were smooth muscle cells and 15–20% were macrophages in balloon-injured atherosclerotic lesions of both ADV-tk/−GC and ADV-tk/+GC rabbits. Atherosclerotic fatty streaks represent proliferative lesions with macrophage foam cells in addition to smooth muscle cells. Both cell types undergo DNA replication in atherosclerotic lesions in uninjured (38) and balloon-injured (22, 39) rabbit arteries. Our results are consistent with previous findings that suggest that the majority of proliferating cells in atherosclerotic lesions are smooth muscle cells but $\sim$ 30% of proliferating cells represent macrophages (38). Our analysis of double-labeled sections indicates a parallel reduction in cell proliferation in both smooth muscle cells and macrophages following tk/GC treatment. These findings extend previous observations to suggest that dividing smooth muscle cells and macrophages are both susceptible to inhibition of cell replication by a cytotoxic agent such as phosphorylated ganciclovir. Furthermore, these findings suggest that genetic strategies to limit cell proliferation in atherosclerotic lesions should be directed towards smooth muscle cells and macrophages, rather than smooth muscle cells alone.

Cell proliferation within the intima and media was limited by this strategy. In this double-injury model, the intima increases by 50% and the media by 20% after the second balloon injury (40). Given the greater degree of expansion and our increased ability to transduce the intima, the antiproliferative effect on intimal area was greater than in the media. Based on the study by Kakuta et al. (40), elimination of the entire portion of intima that results from the secondary injury would result in a decrease in final intimal area of 30–40%, very similar to that found in our study. Combined intimal and medial area was decreased in the ADV-tk/+GC group. This decrease likely represents an inhibition of the expected expansion of the intima and media, rather than a thinning of either structure.

Examination of adenovirus-infected arteries and noninfected arteries and systemic organs revealed no significant differences in arterial inflammation and no evidence of major organ pathology in these rabbits. Despite the presence of some intravascular inflammation in these ADV-transduced arteries (4, 6, 7, 15), this effect will not likely preclude the use of such vectors in clinical settings. While side effects from systemic ganciclovir therapy have been described after long term administration in AIDS patients, including myelosuppression (41, 42), no biological or hematological effects from a 6-d course of treatment were observed in these rabbits. A short course (14 d) of ganciclovir has not been associated with renal, hematological, or other systemic toxicities in human use for cancer gene therapy (43). These findings are also consistent with previous observations of ganciclovir treatment in rats and pigs (4–6), suggesting the relative safety of this prodrug to treat vascular occlusive diseases.

Local expression of a herpesvirus thymidine kinase gene and systemic ganciclovir treatment effectively inhibited cell proliferation and intimal expansion in injured, atherosclerotic rabbit arteries. The degree of inhibition of cell proliferation

and reduction in intimal lesion size achieved by ADV-tk gene transfer in atherosclerotic arteries is comparable to that achieved with cytostatic and cytotoxic approaches (4–7) in nonatherosclerotic arteries. Further studies will determine whether this degree of inhibition of intimal hyperplasia will result in a significant increase in arterial blood flow in the setting of restenosis. Finally, these findings suggest that the presence of atherosclerosis is not a limitation to effective arterial gene transfer. This prodrug strategy may provide a genetic approach to the treatment of restenosis and other vascular proliferative diseases.

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References

1. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.).* 362:801–809.

2. Gibbons, G.H., and V.J. Dzau. 1994. The emerging concept of vascular remodeling. *N. Engl. J. Med.* 330:1431–1438.

3. Libby, P. 1995. Molecular bases of the acute coronary syndromes. *Circulation.* 91:2844–2850.

4. Ohno, T., D. Gordon, H. San, V.J. Pompili, M.J. Imperiale, G.J. Nabel, and E.G., Nabel. 1994. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science (Wash. DC).* 265:781–784.

5. Guzman, R.J., E.A. Hirschowitz, S.L. Brody, R.G. Crystal, S.E. Epstein, and T. Finkel. 1994. In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of herpes simplex thymidine kinase gene. *Proc. Natl. Acad. Sci. USA.* 91:10732–10736.

6. Chang, M.W., T. Ohno, D. Gordon, M.M. Lu, G.J. Nabel, E.G. Nabel, and J.M. Leiden. 1995. Adenovirus-mediated transfer of the herpes virus simplex virus thymidine kinase gene inhibits vascular smooth muscle cell proliferation and neointima formation following balloon angioplasty of the rat carotid artery. *Mol. Med.* 1:172–181.

7. Chang, M.W., E. Barr, J. Seltzer, J.-Q. Jiang, G.J. Nabel, E.G. Nabel, M.S. Parmacek, and J.M. Leiden. 1994. Cytostatic gene therapy for vascular proliferative disorders using a constitutively active form of Rb. *Science (Wash. DC).* 267:518–522.

8. Indolfi, C., E.V. Avvedimento, A. Rapacciuolo, E. Di Lorenzo, G. Esposito, E. Stabile, A. Feliciello, E. Mele, P. Guiliano, G. Condorelli, et al. 1995. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. *Nature Medicine.* 1:541–545.

9. Gordon, J.W., G.A. Scangos, D.J. Plotkin, J.A. Barbosa, and F.H. Ruddle. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA.* 77:7380–7384.

10. Borrelli, E.R., R. Heyman, M. Hsi, and R.M. Evans. 1988. Targeting of an inducible toxic phenotype in animal cells. *Proc. Natl. Acad. Sci. USA.* 85: 7572–7576.

11. Culver, K.W., Z. Ram, S. Wallbridge, H. Ishii, E.H. Oldfield, and R.M. Blaese. 1992. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science (Wash. DC).* 256:1550–1552.

12. Chen, S.H., H.D. Shine, J.C. Goodman, R.G. Grossman, and S.L.C. Woo. 1994. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc. Natl. Acad. Sci. USA.* 91: 3054–3057.

13. Feldman, L.J., P.G. Steg, L.P. Zheng, D. Chen, M. Kearney, S.E. McGarr, J.J. Barry, J.-F. Dedieu, M. Perricaudet, and J.M. Isner. 1995. Low-efficiency of percutaneous adenovirus-mediated arterial gene transfer in the atherosclerotic rabbit. *J. Clin. Invest.* 95:2662–2671.

14. French, B.A., W. Mazur, N.M. Ali, R.S. Geske, J.P. Finnigan, G.P. Rodgers, R. Roberts, A.E. Raizner. 1994. Percutaneous transluminal in vivo gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. *Circulation.* 90: 2403–2413.

15. Muller, D.W.M., D. Gordon, H. San, Z.Y. Yang, V.J. Pompili, G.J. Nabel, and E.G. Nabel. 1994. Catheter-mediated pulmonary vascular gene transfer and expression. *Circ. Res.* 75:1039–1049.

16. Isik, F.F., T.O. McDonald, M. Ferguson, E. Yamanaka, and D. Gordon. 1992. Transplant arteriosclerosis in a rat aortic model. *Am. J. Pathol.* 141:1139–1149.

17. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55–63.

18. Gonchoroff, N.J., J.A. Katzman, R.M. Currie, E.L. Evans, D.W. Houck, B.C. Kline, P.R. Greipp, and M.R. Loken. 1986. S-phase detection with an antibody to bromodeoxyuridine. Role of DNase pretreatment. *J. Immunol. Methods.* 93:97–101.

19. Tsukada, T., M.E. Rosenfeld, R. Ross, A.M. Gown. 1986. Immunocytochemical analysis of cellular components in lesions of atherosclerosis in Watanabe and fat-fed rabbits using monoclonal antibodies. *Arteriosclerosis.* 6:601–613.

20. Faxon, D.P., T.A. Sanborn, V.J. Wever, C.C. Haudenschild, S.B. Gottsman, W.A. McGovern, and T.J. Ryan. 1984. Restenosis following transluminal angioplasty in experimental atherosclerosis. *Arteriosclerosis.* 4:189–195.

21. Gellman, J., M.D. Ezekowitz, I.J. Sarembock, M.A. Azrin, L.E. Nochomowitz, E. Lerner, and C.C. Haudenschild. 1991. Effect of lovastatin on intimal hyperplasia after balloon angioplasty: a study in an atherosclerotic hypercholesterolemic rabbit. *J. Am. Coll. Cardiol.* 17:251–259.

22. Hanke, H., T. Strohschneider, M. Oberhoff, E. Betz, and K.R. Karsch. 1990. Time course of smooth muscle cell proliferation in the intima and media of arteries following experimental angioplasty. *Circ. Res.* 67:651–659.

23. Sarembock, I.J., P.J. LaVeau, S.L. Sigal, I. Timms, J. Sussman, C.C. Haudenschild, and M.D. Ezekowitz. 1989. Influence of inflation pressure and balloon size on the development of intimal hyperplasia after balloon angioplasty. A study in the atherosclerotic rabbit. *Circulation.* 80:1029–1040.

24. Nabel, E.G., D. Gordon, Z.-Y. Yang, L. Xu, H. San, G.E. Plautz, B.-Y. Wu, X. Gao, L. Huang, and G.J. Nabel. 1992. Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. *Hum. Gene Ther.* 3:649–656.

25. Simons, M., E.R. Edelman, J.L. DeKeyser, R. Langer, and R.D. Rosenberg. 1992. Antisense c-*myb* oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature (Lond.).* 359:67–70.

26. Morishita, R., G.H. Gibbons, K.E. Ellison, M. Nakajima, L. Zhang, Y. Kaneda, T. Ogihara, and V.J. Dzau. 1993. Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. *Proc. Natl. Acad. Sci. USA.* 90: 8474–8478.

27. Bennett, M.R., S. Anglin, J.R. McEwan, R. Jagoe, A.C. Newby, and G.I. Evan. 1994. Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by C-*myc* antisense oligodeoxynucleotides. *J. Clin. Invest.* 93:820–828.

28. Morishita, R., G.H. Gibbons, K.E. Ellison, M. Nakajima, H. von der Leyen, L. Zhang, Y. Kaneda, T. Ogihara, and V.J. Dzau. 1994. Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. *J. Clin. Invest.* 93:1458–1464.

29. Simons, M., E.R. Edelman, and R.D. Rosenberg. 1994. Antisense proliferating cell nuclear antigen oligonucleotides inhibit intimal hyperplasia in a rat carotid artery injury model. *J. Clin. Invest.* 93:2351–2356.

30. Shi, Y., A. Fard, A. Galeo, H.G. Hutchinson, P. Vermani, G.R. Dodge, D.J. Hall, F. Shaheen, and A. Zalewski. 1994. Transcatheter delivery of c-*myc* antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. *Circulation.* 90:944–951.

31. Villa, A.E., L.A. Guzman, E.J. Poptic, V. Labhasetwar, S. D'Souza, C.L. Farrell, E.F. Plow, R.J. Levy, P.E. DiCorleto, and E.J. Topol. 1995. Effects of antisense c-*myb* oligonucleotides on vascular smooth muscle cell proliferation and response to vessel wall injury. *Circ. Res.* 76:505–513.

32. Bi, W.L., L.M. Parysek, R.J. Warnick, and P.J. Stambrook. 1993. In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV-tk retroviral gene therapy. *Hum. Gene Ther.* 4:725–731.

33. Freeman, S.M., C.N. Abboud, K.A. Whartenby, C.H. Packman, D.S. Koeplin, F.L. Moolten, and G.N. Abraham. 1993. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* 53:5274–5283.

34. Lemarchand, P., M. Jones, I. Yamada, and R.G. Crystal. 1993. In vivo gene transfer and expression in normal uninjured blood vessels using replication-defective recombinant adenovirus vectors. *Circ. Res.* 72:1132–1138.

35. Guzman, R.J., P. Lemarchand, R.G. Crystal, S.E. Epstein, and T. Finkel. 1993. Efficient and selective adenovirus-mediated gene transfer into vascular neointima. *Circulation.* 88:2838–2848.

36. Barr, E., J. Carroll, A.M. Kalynych, S.K. Tripathy, K. Kozarsky, J.M. Wilson, and J.M. Leiden. 1994. Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Ther.* 1:51–58.

37. Steg, P.G., L.J. Feldman, J.-Y. Scoazec, O. Tahlil, J.J. Barry, S. Boulechfar, T. Ragot, J.M. Isner, and M. Perricaudet. 1994. Arterial gene transfer to rabbit endothelial and smooth muscle cells using percutaneous delivery of an adenoviral vector. *Circulation.* 90:1648–1656.

38. Rosenfeld, M.E., and R. Ross. 1990. Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparable hyper-

cholesterolemic fat-fed rabbits. *Arteriosclerosis.* 10:680–687.

39. Stadius, M.L., A.M. Gown, R. Kernoff, and C.L. Collins. 1994. Cell proliferation after balloon injury of iliac arteries in the cholesterol-fed New Zealand White rabbit. *Arterioscler. Thromb.* 14:727–733.

40. Kakuta, T., J.W. Currier, C.C. Haudenschild, T.J. Ryan, and D. Faxon. 1994. Differences in compensatory vessel enlargement, not intimal formation, account for restenosis after angioplasty in the hypercholesterolemic rabbit model. *Circulation.* 89:2809–2815.

41. Cohen, A.J., B. Weiser, Q. Afzal, and J. Fuhrer. 1990. Ventricular tachycardia in two patients with AIDS receiving ganciclovir. *AIDS (Phila.).* 4:807–809.

42. Faulds, D., and R.C. Heel. 1990. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. *Drugs.* 39:597–638.

43. Culver, K.W., and J. VanGilder. 1994. Clinical protocol: gene therapy for the treatment of malignant brain tumors with in vivo tumor transduction with the herpes simplex thymidine kinase gene/ganciclovir system. *Hum. Gene Ther.* 5:343–379.